



## The influence of sex, parasitism, and ontogeny on the physiological response of European eel (*Anguilla anguilla*) to an abiotic stressor

**Silva, Ana T.; Midwood, Jonathan D.; Aarestrup, Kim; Pottinger, Tom G.; Madsen, Steffen S.; Cooke, Steven J.**

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Contact CEH NORA team at  
[noraceh@ceh.ac.uk](mailto:noraceh@ceh.ac.uk)

1    **THE INFLUENCE OF SEX, PARASITISM, AND ONTOGENY ON THE**  
2    **PHYSIOLOGICAL RESPONSE OF EUROPEAN EEL (*Anguilla anguilla*)**  
3    **TO AN ABIOTIC STRESSOR**

4    *Short title: Interaction of biotic and abiotic factors on stress response of eels*

6    **Ana T. Silva<sup>1,2\*</sup>, Jonathan D. Midwood<sup>2,3</sup>, Kim Aarestrup<sup>4</sup>, Tom G. Pottinger<sup>5</sup>, Steffen S.**  
7    **Madsen<sup>6</sup>, Steven J. Cooke<sup>2</sup>**

9    <sup>1</sup>*NINA-The Norwegian Institute for Nature Research, Trondheim, Norway*

10    <sup>2</sup>*Fish Ecology and Conservation Physiology Laboratory, Department of Biology, Carleton University,*  
11    *Ottawa, Ontario, K1S 5B6, Canada,*

12    <sup>3</sup>*Great Lakes Laboratory for Fisheries and Aquatic Science, Fisheries and Oceans Canada, Burlington,*  
13    *Ontario, L7S 1A1, Canada,*

14    <sup>4</sup>*National Institute of Aquatic Resources, Freshwater Fisheries, Technical University of Denmark,*  
15    *Vejlsøvej 39, 8600 Silkeborg, Denmark*

16    <sup>5</sup>*Centre for Ecology and Hydrology, Lancaster Environment Centre, Library Avenue, Bailrigg,*  
17    *Lancaster LA1 4AP, United Kingdom*

18    <sup>6</sup>*Department of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M,*  
19    *Denmark*

21    \*Corresponding author. Email: [ana.silva@nina.com](mailto:ana.silva@nina.com), [anamftsilva@gmail.com](mailto:anamftsilva@gmail.com)

23    **What is already known:**

24    The consequences of different biotic factors and their interaction on the physiological stress  
25    response of eels to abiotic stressors have long been assumed. Yet, very few studies have  
26    explored these relationships using empirical research. Such information is crucial to develop  
27    effective management practices needed to assist with the recovery of the European eel, currently  
28    classified as an Endangered species.

29    **What this study adds:**

30    This study revealed the importance of considering the role of biotic factors (in this case: sex,  
31    parasitism and ontogeny) acting together to influence the stress response of the European eel to  
32    abiotic stressors. To our knowledge this is the first physiological study that simultaneously  
33    examines these different biotic factors. Furthermore, this study is highly relevant as there is a  
34    paucity of information on the influence of biotic factors on the physiological response of the  
35    European eel and other fish species to different abiotic stressors.

## ABSTRACT

Migration of adult European eel (*Anguilla anguilla*) from freshwater feeding grounds to oceanic spawning grounds is an energetically demanding process and is accompanied by dramatic physiological and behavioural changes. Humans have altered the aquatic environment (e.g. dams) and made an inherently challenging migration even more difficult; human activity is regarded as the primary driver of the collapse in eel populations. The neuroendocrine stress response is central in coping with these challenging conditions, yet, little is known about how various biotic factors such as sex, parasites, and ontogeny influence (singly and via interactions) the stress response of eel. In this study, mixed effect models and linear models were used to quantify the influence of sex, parasitism (*Anguillicola crassus*), life-stage (yellow and silver eels), and silvering stage on the stress response of eels when exposed to a standardized handling stressor. The physiological response of eels to a standardized abiotic stressor (netting confinement in air) was quantified through measurements of blood glucose and plasma cortisol. The relationships between biotic factors and the activity of gill  $\text{Na}^+/\text{K}^+$  - ATPase was also examined. Analyses revealed that in some instances a biotic factor acted alone while in other cases several factors interacted to influence the stress response. Blood glucose concentrations increased following exposure to the standardized stressor and remained elevated after 4 hours. Variation in plasma cortisol concentrations following exposure to the stressor were found to be time-dependent, which was exacerbated by the life-stage and parasitism condition. Males and non-parasitized silver eels had the highest  $\text{Na}^+/\text{K}^+$ -ATPase activity. Silvering stage was strongly positively correlated with  $\text{Na}^+/\text{K}^+$ -ATPase activity in female eels. Collectively, these findings confirm that the factors mediating stress responsiveness in fish are complicated and aspects of inherent biotic variation cannot be ignored.

- 61    **Keywords:** silver eel, yellow eel, stress response, *Anguillicola crassus*, cortisol, glucose,
- 62     $\text{Na}^+/\text{K}^+$ -ATPase activity

## INTRODUCTION

In freshwater and marine ecosystems fish are often exposed to natural and anthropogenic stressors (Arthington et al. 2016). To compensate for the challenge imposed by a stressor, fish undergo a series of biochemical and physiological changes (i.e., the stress response; Wendelaar Bonga 1997; Gorissen, and Flik 2017). The glucocorticoid stress response is an essential mediator of allostasis that maintains stability (homeostasis) or facilitates adaptation to changing conditions (McEwen and Wingfield 2003; Angelier 2013), therefore promoting the survival and recovery of individuals (Sapolsky et al. 1999). The stress response is characterized by the production and release of glucocorticoid steroid hormones (i.e., cortisol in fish) shortly after the perception of the stressor (Axelrod and Reisine 1984). In the short term, this stress response is adaptive, providing the fuel (i.e., glucose) needed to respond to a stressor (Mommsen et al. 1999; Barton 2002). However, if the stressor persists, the action of glucocorticoids can occur at the expense of other life-history components through a reduction in the amount of energy available for essential functions (Korte et al. 2005). In fish, stress can negatively affect growth, health (immunocompetence), reproduction, and welfare, and ultimately result in mortality (Schreck 1981, 2000; Barton 2002; Fuzzen et al. 2011).

For diadromous fish species, the transition from life in freshwater (FW) to seawater (SW) is a very important and a challenging period usually characterized by high levels of mortality (Bruijs et al. 2009; Piper et al. 2015). The European eel (*Anguilla anguilla*), a catadromous species, undertakes an outward migration of ~5000–6000 km to spawning grounds in the Sargasso Sea (van Ginneken et al. 2005; Aarestrup et al. 2009), which is known as the longest spawning migration among all the species of eels (Aoyama 2009) and is performed without feeding (Righton et al. 2012). Before migrating to SW eel's life is spent feeding in freshwater (for up to 25 years) to store enough fat (>20% of the body mass; Tesch 2003) (yellow eel stage) to fuel migration that may take many months (Righton et al. 2016), as well as, to provide

sufficient energy to produce offspring. After attaining an adequate lipid reserve, eels start lipid mobilization (EELREP 2005; Trischitta et al. 2013) and sexual maturation, metamorphosing into “silver eels”. During this stage, eels stop feeding, and begin the long migration back to the Sargasso Sea for spawning (Righton et al. 2012). Males (on average 40 cm) usually start their migration in August while females (on average bigger than 40 cm) leave later, during October and December (Tesch 2003).

Spawning migration of eels is a complex and energetically demanding process during which eels are very vulnerable to natural and anthropogenic challenges that can impair their migratory capacity as they transition from freshwater to saline water (Gollock et al. 2005, Iversen et al. 2013, Trischitta et al. 2013, Wilson 2013). Durif et al. (2005) described five different stages of the silvering process in female eels according to their physiological changes as they prepare for their spawning migration: a growth phase (I and II) a pre-migration phase (II) and two migration phases (IV and V). In part due to their catadromous lifestyle, European eel populations have seen marked declines throughout their natural range in the past few decades and are currently classified as Critically Endangered (Jacoby and Gollock 2014) and listed under Appendix I-III of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES 2013). Several factors are thought to have contributed to these declines including barriers to migration, habitat loss, parasites (e.g. *Anguillicola crassus*), disease, climate change, bioaccumulation of toxins, predation, changes in ocean currents and overfishing (Dekker 2003; Knights 2003; Van Ginneken et al. 2005; Belpaire et al. 2009; Geeraerts and Belpaire 2010; Durif et al. 2011; Kettle et al. 2011; Wahlberg et al. 2014). The drastic decline of European eel populations has hastened the implementation of management measures aimed at restoring stocks by preventing mortality during migration (European Union implemented the Eel Recovery Plan 2007- Council Regulation No. 1100/2007/EC and the International Council for the Exploitation of the Sea -ICES 2014).

Despite the extensive body of literature that has explored the stress response of fish in general (reviewed in Schreck 2010; Pankhurst 2011), to our knowledge no studies have specifically explored how biotic characteristics acting in concert may influence the stress response and recovery in European eel, as analysed in this study. The main goal of this study was to analyze how individual factors such as sex, parasitic load (non-parasitized and parasitized with *A. crassus*), and ontogenetic phase (yellow, silver, and different silvering stages) interact to influence the physiological response to a standardized handling and air exposure stressor. To determine which biotic characteristics are associated with the stress response, we used mixed effect and linear models to quantify the physiological responses of eels. We measured blood parameters (i.e., plasma cortisol and body glucose) immediately (baseline), 1 hour (stress response) and 4 hours (recovery period) post-exposure to the stressor. We also tested for relationships between biotic factors and the activity of gill  $\text{Na}^+/\text{K}^+$  - ATPase given the important role of this gill enzyme in diadromous species. Moreover, plasma cortisol is also associated with branchial  $\text{Na}^+/\text{K}^+$ -ATPase activity, which plays a central role in whole-body osmoregulation (Towle 1981; Sancho et al 1997) such that stress has the potential to also influence osmoregulatory processes.

## **MATERIAL AND METHODS**

### *Animals and experimental design*

European eels were caught during downstream migration between October and November of 2014 in a trap located in River Gudenå at Vestbirk hydropower station, at a downstream trap in Flade Sø and by electrofishing at Bygholm Å and Lake Stigsholm, Denmark. The eels ( $N=72$ , mean total length ( $L_t$ )  $\pm$  S.D. =  $51.9 \pm 8.3$  cm, mean total weight ( $W_t$ )  $\pm$  S.D. =  $249.7 \pm 127.3$  g) were transported and held in three 8000L holding tanks (water temperature 12-15°C) at the National Institute of Aquatic Resources, Technical University of Denmark, in Silkeborg,



Denmark, until the experiments were carried out (holding time of between 5 and 9 days). To minimize stress during holding and facilitate recovery from capture, transportation and handling, shelter was provided for the eels. This shelter was comprised of 3.0 and 4.5 cm diameter by 70 cm long PVC pipes that were placed in the holding tanks. These pipes also limited the influence of removal of an individual for treatment on the remaining eels in the holding tank since a single pipe could be removed without disturbing the other eels. Overall, 57 females eels (mean  $L_t \pm S.D.$  =  $54.9 \pm 6.1$  cm, mean  $W_t \pm S.D.$  =  $289.7 \pm 111.9$  g) and 15 males (mean  $L_t \pm S.D.$  =  $40.5 \pm 3.4$  cm, mean  $W_t \pm S.D.$  =  $105.13 \pm 27.2$  g), were tested. Each eel received the same experimental treatment. First, an eel was removed from the holding tank by netting a PVC pipe on either end and lifting it from the tank, with minimal disturbance. A blood sample was then collected within 3-min of capture to act as baseline sample of plasma cortisol and blood glucose (as per Lawrence et al. 2018). Next, the eel was exposed to a standardized stressor in the form of a 10-min air exposure, before being moved into an individual 80-L holding tank with 20-L of water. To measure the magnitude of the stress response in each eel, blood samples were collected again at one and four hours after their baseline sample. Eels were not anaesthetised during this procedure because it has been shown to influence gill  $Na^+/K^+$ -ATPase activity (Toni et al. 2014) – another parameter measured in this study (details provided in the *Plasma and Gill sample analysis* section) and would have confounded our ability to measure the stress response. Anesthesia can influence the stress response in a number of ways – both muting it and also serving as a stressor itself (there is a significant metabolic demand associated with clearing anesthetics; Neiffer and Stamper 2009). We acknowledge that the blood sampling at the 1 hr time point would have served as a stressor that had the potential to influence the stress levels measured at the 4 hr time point but all fish were handled similarly and this occurred during a period when the stress response was already at its peak. Stress associated with sampling during the first blood sampling period was simply

part of the standardized stressor while stress associated with sampling during the final time point was irrelevant given that no further sampling would occur. Blood sampling without anaesthesia is relatively common in the study of stress physiology in wild fish (e.g., Cooke et al., 2005) including studies that involve repeated sampling of individuals (e.g., Cook et al. 2012). To minimise disturbance of fish during blood sampling, this procedure was always conducted by the same operator. Fish were euthanized via decapitation using a sharp knife. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Animal care approval for this study falls under the Danish Animal Experiment Inspectorate (licence number: 2013-15-2934-00808).

#### *Individual condition*

At the end of each experiment eels were sacrificed and measured for body mass, total length, body width at maximum body depth, body height at maximum body depth, pectoral fin length and horizontal and vertical eye diameters. These measurements were used to distinguish males from females and to calculate three morphometric indices: eye index, fin index and Fulton's condition factor (Durif et al. 2005; Bolger and Connolly 1989). These indices were used together with the external morphological characteristics of silver-phase eel (presence of black corpuscles in the lateral line; dark dorsal part of the body and lighter “silver” ventral region; and snout shape and dark coloration of the extremities of the pectoral fins and tail), as selective criteria to distinguish between the yellow and silver phases, as well as to determine the different silvering stage (stage I to V; Pankhurst 1982; Durif et al. 2005). The swimbladder of each eel was also removed and any *A. crassus* present in the swimbladder lumen were removed and enumerated.

#### *Plasma and Gill sample analysis*

Blood samples were obtained by puncture of the caudal vasculature using pre-heparinised (10 000 USP units/ml heparin sodium: Sandoz, Canada), needles (25 G 1/2”) and 1 ml syringes (BD Plastipak, 1ml) and the blood was stored briefly in ice. The total sampling time never exceeded 3 min. The volume of blood removed for each sample was approximately 0.2 ml. After each blood sample was obtained, sub-samples were removed for immediate determination of blood glucose concentrations using a glucose meter (Accucheck, Roche Diagnostics; Stoot et al. 2014) and the remainder of the sample was centrifuged for 10 min at 4,000 RPM to separate plasma from the blood cells. The aliquoted plasma was immediately frozen in liquid N<sub>2</sub> and then stored frozen at –80°C for later analysis. Individual plasma cortisol concentrations (ng/mL) were determined according to the radioimmunoassay procedure described in Pottinger and Carrick (2001) with two minor adjustments. The antibody used in this study was IgG-F-2 rabbit anti-cortisol (IgG Corp; Nashville, TN, USA) and tracer ([1,2,6,7]<sup>3</sup>H-cortisol, 2.59 TBq/mmol; Perkin-Elmer, U.K.) was added in a 25 µL aliquot of buffer at the same time as the antibody was dispensed.

Measurement of gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity followed procedures outlined by McCormick (1993). Gill filaments from the second right gill arch were removed from each eel, placed in a tube containing ice-cold SEI buffer (300 mM sucrose, 20 mM Na<sub>2</sub>EDTA, 50 mM imidazole, pH 7.3) frozen in N<sub>2</sub> and stored at –80°C until analysed. Gill homogenates were centrifuged at 1000 g for 1 min and the supernatant was assayed for ATPase activity in the presence and absence of 0.5 mM ouabain. Each assay was run in triplicate. Protein content was measured by the Lowry (1951) method modified for a plate reader. The difference between the two determined activities (with and without ouabain) was calculated as the Na<sup>+</sup>/K<sup>+</sup>-ATPase activity.

### *Statistical Analysis*

Data were analysed for normality using the Shapiro-Wilcoxon test. To meet the normality requirements of parametric analysis, cortisol and glucose data were log(x) transformed (log-cortisol (logC) and log-glucose (logG), respectively).

Response variables logC and logG were fitted with linear mixed effects models (LME) with individual fish as a random factor and time (baseline, 1 hour and 4 hours), sex (male or female), life-stage (yellow or silver) and parasite condition (non-parasitized vs parasitized with *A. crassus*) as fixed effects. Silvering stages (I to V) could not be compared independently due to the small number of individual females in each stage; therefore, individuals were grouped in three groups according to their similarities of development (after Durif et al. 2005). Group 1 included all the individuals belonging to the silvering stage I and II, group 2 had individuals in stage III and group 3 had individuals in stage IV and V. To understand the effects of silvering stage on logC and logG, a new LME model was run with silvering condition included as a fixed effect and sex and stage (redundant factor) removed as possible predictors. Only females (N=57: silver N=35; yellow N=22) were used in this analysis as the number of silver males was very low for a statistical analysis (N=15: silver N=6; yellow N=9).

Linear models (LM) were used to assess the effect of sex, life-stage, parasite condition and silvering stages on gill  $\text{Na}^+/\text{K}^+$ -ATPase activity. Data were analysed using the *nlme* function implemented in the R statistical environment (package version 3.1-117, R core team ; Pinheiro et al, 2017). To compare model fits objectively, and determine which was the most appropriate, an information theoretic approach was performed to compare models using Akaike's information criterion (AIC; Akaike 1974; Burnham and Anderson, 2002). Models were validated by examining histograms of the normalized residuals, plotting the normalized residuals against fitted values. The final models were refitted using maximum likelihood (ML). Mean values are reported together with standard error (mean  $\pm$  S.E) and results were considered significant for  $\alpha < 0.05$ .

## RESULTS

### *Parasitism*

Overall, 20 eels were parasitized with *A. crassus*. The number of parasites in the eels varied between 1 and 11 individuals per specimen, and it was different according to the : sex (females: N= 19, males N=1), life-stage (yellow eels N= 7, silver eels N=13) and silvering condition (life stage I: N= 7, life stage II, N= 8, Life stage III: N= 4). The different number of parasites in each silvering stage resulted in different levels of Glucose, Cortisol and Gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity (Table 1).

### *Glucose*

The final model for blood glucose (logG) contained time and parasite condition as the main explanatory factors (logG~time\*parasitism; AIC : -222.05, dF: 8). Blood glucose varied significantly with time (Table 2), and increased from  $4.5 \pm 0.3$  mmol/L (mean  $\pm$  S.E) in unstressed eels at 0 h to  $7.6 \pm 0.4$  mmol/L at 1 hour after the stressor and to  $10.2 \pm 0.5$  mmol/L 4 hours following the stressor (Fig. 1). Temporal variation of glucose was similar between parasitized and non-parasitized eels, with parasitized eels exhibiting slightly higher overall glucose levels ( $7.5 \pm 0.4$  mmol/L) than non-parasitized eels ( $7.1 \pm 0.3$  mmol/L; Fig. 1), although this difference was not significant.

Variation of plasma glucose in female eels was best explained by a model that included both time and the interaction of parasite condition with silvering (logG~time+parasitism\*silvering; AIC: -145.53, dF: 8). Plasma glucose levels at 1h ( $7.7 \pm 0.5$  mmol/L) and 4h ( $10.2 \pm 0.6$  mmol/L) after the stressor were significantly different from the values in unstressed eels ( $4.5 \pm 0.4$  mmol/L) (Table 2). Parasitism and life-stage were also important covariates in explaining the variation of plasma glucose in female eels, improving

the statistical model, nevertheless their effects were not statistically significant (Table 2). Indeed, similar number of parasites in each silvering stage led to approximately the same values on plasma glucose on eels. Although, minor, still, blood glucose increased the number of *A. crassus* existent in each eel, in particularly in eels parasitized with more than 4 individuals. (Table 1). Overall, mean glucose levels in non-parasitized female eels were lower ( $6.8 \pm 0.4$  mmol/L) than those in parasitized female eels ( $7.9 \pm 0.4$  mmol/L).

#### *Plasma cortisol*

Plasma cortisol levels varied significantly with time (Table 2) and were also dependent on parasite condition and life stage of eels ( $\log C \sim \text{time} * \text{parasitism} + \text{parasitism} * \text{life-stage}$ ; AIC: 29.42, dF: 10). Mean plasma cortisol levels significantly increased in the first hour after the stressor from  $29.19 \pm 4.0$  ng/mL to  $57.84 \pm 3.48$  ng/mL after which they decreased to levels slightly higher than those in unstressed eels ( $37.73 \pm 3.6$  ng/mL) (Table 2). Although non-parasitized eels exhibited higher levels of cortisol overall ( $48.6 \pm 3.8$  ng/mL) than parasitized eels ( $33.1 \pm 2.4$  ng/mL) (Table 2), net changes in variation was larger in parasitized eels (Fig. 2a). This was particularly evident in the first hour where mean plasma cortisol concentrations rose significantly from baseline levels of  $16.9 \pm 2.0$  ng/mL to  $54.2 \pm 3.2$  ng/mL (Table 2, Fig. 2a). Overall, non-parasitized silver eels had higher plasma cortisol levels ( $58.6 \pm 6.7$  ng/mL) when compared to non-parasitized yellow eels ( $39.15 \pm 3.5$  ng/mL). Nonetheless, parasitism strongly influenced cortisol response in silver eels, which had the lowest levels of cortisol found ( $30.7 \pm 2.5$  ng/mL) (Fig. 2b, Table 2).

In female eels, plasma cortisol concentrations were found to vary with silvering stage and the interaction between time and parasitism ( $\log C \sim \text{silvering} + \text{time} * \text{parasitism}$ ; AIC : 36.06, dF: 10). Female eels belonging to the maximum silvering stage group exhibited higher levels of plasma cortisol ( $58.02 \pm 6.3$  ng/mL), when compared to eels of the second ( $27.9 \pm 2.3$  ng/mL)

and first group ( $35.5 \pm 3.3$  ng/mL) (Table 2). Parasitized female eels exhibited the lowest levels of plasma cortisol ( $32.3 \pm 2.8$  ng/mL) when compared to non-parasitized eels ( $49.1 \pm 5.0$  ng/mL). The variation of plasma cortisol in female parasitized eels was found to increase with the number of *A. crassus* (Table 1). When parasitized with more than 4 individuals eels had an increased on plasma cortisol levels. Nevertheless when in the last silvering stage, even a small number *A. crassus* appears to elicit a strong increase of plasma cortisol. Variation in plasma cortisol was also time dependent (Table 2); plasma cortisol significantly increased from  $27.9 \pm 5.0$  ng/mL to  $56.5 \pm 4.1$  ng/mL in the first hour following the stressor, decreasing to values close to the baseline levels after 3h ( $38.6 \pm 4.5$  ng/mL) (Table 2). This temporal variation was found to be related to the parasitism status of the individual (Table 2). After exposure to a stressor, parasitized eels exhibited a stronger increase in cortisol levels when compared to non-parasitized eels (Table 2). This variation was clearly evident in the first hour following disturbance (non- parasitized eels:  $61.9 \pm 7.1$  ng/mL, parasitized eels:  $53.9 \pm 3.5$  ng/mL) (Table 2). However, by the 4h time point, plasma cortisol levels had recovered to near the levels seen in non-parasitized eels (4h:  $44.4 \pm 8.0$  ng/mL, baseline:  $39.9 \pm 9.7$  ng/mL), but not in parasitized eels (4h:  $26.2 \pm 3.0$  ng/mL, baseline:  $14.7 \pm 2.2$  ng/mL) (Table 2).

#### *Gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity*

Gill Na<sup>+</sup>/K<sup>+</sup>-ATPase activity varied between individuals of different sexes, life stages and parasitism levels (Na<sup>+</sup>/K<sup>+</sup>-ATPase activity~ sex + life-stage\*parasitism; AIC: 293.97, dF: 6). Males exhibited higher levels of Na<sup>+</sup>/K<sup>+</sup>-ATPase ( $8.71 \pm 0.8$  μmol ADP/mg protein/h) than females ( $6.02 \pm 0.5$  μmol ADP/mg protein/h) (Table 2). Na<sup>+</sup>/K<sup>+</sup>-ATPase levels were found to vary with the life-stage of eels, with the highest values found in silver eels ( $7.97 \pm 0.6$  μmol ADP/mg protein/h) when compared to yellow eels ( $4.74 \pm 0.5$  μmol ADP/mg protein/h) (Fig. 3, Table 2). Within life stages the variation of Na<sup>+</sup>/K<sup>+</sup>-ATPase activity was conditioned by the

parasitism level, particularly in silver eels where non-parasitized individuals exhibited significantly higher  $\text{Na}^+/\text{K}^+$ -ATPase activity ( $10.26 \pm 0.8 \mu\text{mol ADP/mg protein/h}$ ) than parasitized silver eels ( $7.22 \pm 0.5 \mu\text{mol ADP/mg protein/h}$ ) (Fig. 3, Table 2).

In female eels, gill  $\text{Na}^+/\text{K}^+$ -ATPase activity increased through silvering stage (Table 2). The highest values of  $\text{Na}^+/\text{K}^+$ -ATPase activity were found in the third silvering group ( $7.74 \pm 0.8 \mu\text{mol ADP/mg protein/h}$ ), decreasing in the second group ( $5.79 \pm 0.6 \mu\text{mol ADP/mg protein/h}$ ) and were lowest in the first group ( $4.02 \pm 0.5 \mu\text{mol ADP/mg protein/h}$ ). Gill  $\text{Na}^+/\text{K}^+$ -ATPase activity in female parasitized eels increased with the number of *A. crassus*, in particular in eels parasitized with more than 4 *A. crassus* (Table 1).

## DISCUSSION

In this study we examined the effect of sex, parasite burden, and ontogeny, alone and in combination, on the stress response and  $\text{Na}^+/\text{K}^+$ -ATPase activity of European eels when exposed to a standardized handling stressor. To our knowledge, this is the first study to examine the impact of the interaction of different biotic factors on the physiological response of eels. The results of this study revealed a physiological response to our experimental handling stressor with the extent of the response modulated by biotic factors. Interestingly, in some instances biotic factors acted alone while in other cases several factors interacted to influence the physiological response.

Eels subjected to the stressor exhibited significantly higher concentrations of glucose throughout the 4 h duration of the study, with the most significant increase observed during the first hour after disturbance. The prolonged elevation of glucose reflects a mobilisation of energy to provide short-term support for immediate coping activities to promote survival. Parasitized and non-parasitized eels showed similar levels of glucose, a result consistent with Gollock et al. (2004) and their study on parasite-mediated stress responses to handling stressors in



European eel. Moreover, we observed that the number of *A. crassus* in female parasitized eels led to slightly higher concentrations of blood glucose, but this was not significantly different between the three silvering stages.

As expected, eels also exhibited a strong cortisol response to stress. Cortisol significantly increased in the first hour after exposure to the stressor followed by a decrease in the next 3 hours. This is noteworthy given that we repeatedly sampled fish such that there would have been some level of stress associated with blood sampling at the 1 hour time point. Despite that, cortisol recovery was still evident at the 4 hour time point. When considering both males and females, the temporal variation in cortisol was similar in both parasitized and non-parasitized eels; parasitized eels exhibited a stronger response in terms of increment of cortisol when compared to non-parasitized eels. This finding suggests that parasitic state plays an important role in the stress response of eels. The similarity on the variation and levels of cortisol between this study and Gollock et al. (2004), as well as, the fact that eels used in this study were also wild and may have been infected by *A. crassus* for a long period of time, support the argument of Gollock et al. (2004) that the results obtained can reflect an adaptation to the effects of chronic parasitism. Moreover, Sures et al. (2001) found that there is a strong stress response of eels to the larval and young adult stages of *A. crassus*, but no chronic response to older adults. Although we have not analyzed the life stage of *A. crassus* infecting the tested specimens, it is possible that the tested eels could have been in an early onset of infection. The environmental characteristics of the system where eels lived (water temperature, water salinity) also played an important role on the results obtained as it is known that the spread, extent and intensity of infestation by *A. crassus* is dependent on water salinity and the age and size of the fish (Sures et al. 2001; Lefebvre and Crivelli 2012). Differences in plasma cortisol levels between non-parasitized and parasitized specimens was strongly evident on female silver eels, even if overall there were no significant differences were found between the two life-stages. This evidence that

there is a synergistic influence of multiple stressors on the stress response. Female eels categorized as being in the last stage of silvering (III) exhibited the highest levels of plasma cortisol which may have some implications for reproductive function. Moreover, eels on the third silvering stage were found to be more susceptible to the presence of parasites, as the highest levels of plasma cortisol were found even when the number of parasites was low (<4 individuals). Parasitism on the last silvering stage may negatively influence migration and reproduction of the eels.

High levels of cortisol for prolonged periods of time have been shown to play a role in energy mobilization as result of its lipolytic effect increasing free fatty acid levels, reduce growth rate by increasing the pituitary gonadotropin, reduce immune function, and disrupt fish reproduction function by depressing sex steroid levels (Huang et al. 1999). The implications of high levels of cortisol for prolonged periods during exposure to chronic or frequent intermittent acute stressors on eel reproduction are therefore potentially important. The morphological and physiological transformation of yellow eels to the silver phase and the initiation of their spawning migration is only triggered when the levels of lipids is >20% of the body mass (Palstra et al. 2010; van den Thillart et al. 2009). As such, elevations in cortisol have the potential to influence both maturation and spawning migrations. Cortisol is also known to be related to SW adaptation of fish helping them to acclimate to a hyperosmotic environment (SW) by increasing hypoosmo-regulatory capacity (Mommsen et al. 1999). Cortisol mediates SW-acclimation by stimulating the gill chloride cell proliferation and  $\text{Na}^+/\text{K}^+$ -ATPase activity ensuring the transmembrane transfer of the cations  $\text{Na}^+$  and  $\text{K}^+$  and affecting the transepithelial movements of cations in gills (Madsen 1990a; Sancho et al 1997; McCormick 1995). The stimulatory role of cortisol on gill  $\text{Na}^+/\text{K}^+$ -ATPase activity in the American eel (*Anguilla rostrata*) was previously shown by Butler et al. (1972), on their study of the effects of environmental salinity and adrenocortical steroids on  $\text{Na}^+/\text{K}^+$ -ATPase activity. Also, studies on salmonids showed

that gill  $\text{Na}^+/\text{K}^+$ -ATPase activity responds positively to injections of cortisol in Atlantic salmon, *Salmo salar* (Bisbal and Specker 1991), rainbow trout, *Oncorhynchus mykiss* (Madsen 1990a), and sea trout *Salmo trutta* (Madsen 1990b; Fontaínhas-Fernandes et al. 2003).

Gill  $\text{Na}^+/\text{K}^+$ -ATPase activity was significantly higher in silver eels than in yellow eels. This result was particularly evident in non-parasitized silver eels since parasitized silver eels appeared to have suppressed  $\text{Na}^+/\text{K}^+$  - ATPase activity. Despite the low values of  $\text{Na}^+/\text{K}^+$  - ATPase activity in female parasitized eels, it was observed that these values increased with silvering stage as well as with the number of *A. crassus*. It is known that  $\text{Na}^+/\text{K}^+$ -ATPase activity plays a crucial role in the osmoregulation of eels, thus the suppression of such protein will limit the success of eels in salt water and therefore compromise their migration, reproduction and concomitant survival. Control and mitigation of the levels of *A. crassus* in eels, in particularly on the latest stages of maturation of this species are critical and must be developed. Such conservation measures will contribute to the reduction of the decline of the European eel, currently classified as critically. Considering the well-known effects of cortisol on  $\text{Na}^+/\text{K}^+$ -ATPase activity (McCormick, 1995), the highest levels of this parameter on non-parasitized silver eels may have been related to the high levels of cortisol found in these specimens. Furthermore, non-parasitized yellow eels exhibited the lowest levels of gill  $\text{Na}^+/\text{K}^+$ -ATPase activity. Once again, parasitism acting synergistically with other biotic factors affect ion regulation via an indirect effect on gill  $\text{Na}^+/\text{K}^+$  regulation.

The highest levels of  $\text{Na}^+/\text{K}^+$  - ATPase activity were found in males and can be a consequence of different stages of sexual maturation achieved by the specimens. Considering that males initiate their migration earlier than females (Palstra et al. 2007; 2010), and that the experiments were carried out at the end of October through the beginning of November, our findings may then be related with the fact that most males could have been in a more advanced silvering stage than the females. Although expected, this is an interesting result as it indicates

that the success of spawning of males become more susceptible and/or compromised by environmental conditions (e.g. parasite load) earlier than females. Gill  $\text{Na}^+/\text{K}^+$  - ATPase activity increased with silvering stage. Nevertheless, this variation was exacerbated in non-parasitized eels, which exhibited an elevation of Gill  $\text{Na}^+/\text{K}^+$  - ATPase activity between silvering stage 2 and 3. Again, this points toward the idea that parasitized eels can be adapted to deal with stress, and therefore their response to stress would be less severe.

## CONCLUSIONS

This paper documented a strong glucose and cortisol response of European eels to a holding stressor (netting confinement in air) that was mediated by the interaction of several biotic factors. Such biotic interactions were also found to play an important role in the variation of  $\text{Na}^+/\text{K}^+$ -ATPase activity. Because we assessed the role of multiple biotic factors simultaneously we had the ability to test their influence alone and in combination which is a robust approach relative to examining them individually (e.g., just parasite burden) which has been the typical approach in the literature thus far. Indeed, we revealed that the stress response of eels was found to differ between life stage, sex and parasitism condition, as well as, with the number of parasites. Parasitism, mainly when acting together with other biotic stressors, plays an important role in the physiological response of the eels to stressors and presumably has the potential to influence the maturation, reproductive and osmoregulatory processes in this species. Future studies that examine the influence of biotic factors acting alone and interacting under different abiotic conditions are needed to better understand the role of stress in the different life stages, sex, health conditions and other physiological characteristics of wild fish.

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## FIGURE LEGENDS

Figure 1: Variation of glucose on non- parasitized and parasitized eels through time (baseline, 1 and 4 hours) (mean  $\pm$  standard error).

Figure 2: Variation of plasma cortisol on non- parasitized and parasitized eels: (a) among time (baseline, 1 and 4 hours) (mean  $\pm$  standard error) and (b) between life-stages (yellow and silver). The solid black line represents the median (50th percentile) and the bottom and top box edges are the 25th (Q1) and 75th (Q3) percentile, respectively. The bottom whisker is the  $\max(\min(x), Q1 - 1.5 * IQR)$  with  $IQR = Q3 - Q1$ , whereas the top whisker is the  $\min(\max(x), Q3 + 1.5 * IQR)$ .

Figure 3: Gill  $\text{Na}^+/\text{K}^+$ -ATPase activity variation of non-parasitized and parasitized yellow and silver eels. The solid black line represents the median (50th percentile) and the bottom and top box edges are the 25th (Q1) and 75th (Q3) percentile, respectively. The bottom whisker is the  $\max(\min(x), Q1 - 1.5 * IQR)$  with  $IQR = Q3 - Q1$ , whereas the top whisker is the  $\min(\max(x), Q3 + 1.5 * IQR)$ .

**TABLES**

Table 1. Glucose, Cortisol and Na<sup>+</sup>/K<sup>+</sup>-ATPase activity on female parasitized eels in different silvering conditions according parasite range (mean  $\pm$  standard error).

Silvering stage	Parasite range	N	Variable		
			Cortisol	Glucose	Na <sup>+</sup> /K <sup>+</sup> -ATPase activity
I	(0,4]	5	13.04 $\pm$ 2.69	4.08 $\pm$ 1.07	4.76 $\pm$ 0.63
	(4,8]	2	26.60 $\pm$ 13.30	4.35 $\pm$ 1.65	3.53 $\pm$ 1.02
II	(0,4]	4	9.60 $\pm$ 3.81	4.15 $\pm$ 0.57	7.06 $\pm$ 0.50
	(4,8]	3	11.90 $\pm$ 1.83	3.96 $\pm$ 0.94	4.89 $\pm$ 1.80
	(8,12]	1	14.80 $\pm$ -	4.30 $\pm$ -	7.56 $\pm$ -
III	(0,4]	3	21.53 $\pm$ 8.38	4.60 $\pm$ 1.80	7.95 $\pm$ 1.42
	(4,8]	1	7.80 $\pm$ -	9.60 $\pm$ -	10.05 $\pm$ -

**TABLES**

Table 2. Statistical outputs from linear mixed effects models: random effects model (Glucose and Cortisol) and fixed-effects ( $\text{Na}^+/\text{K}^+$ -ATPase activity). *P* values of significant parameters are indicated.

Variables	Parameter	Value	Std.Error	t-value	p-value
Glucose					
a) General					
	Time (1h)	0.269	0.022	12.165	<0.0001
	Time (4h)	0.410	0.022	18.356	<0.0001
	Parasitized	0.060	0.048	1.239	0.220
	Time (1h) x Parasitized	0.018	0.034	-0.520	0.604
	Time (4h) x Parasitized	0.041	0.034	-1.186	0.238
b) Females in different silvering stages					
	Time (1h)	0.260	0.019	13.352	<0.0001
	Time (4h)	0.388	0.020	19.747	<0.0001
	Parasitized	0.044	0.088	0.511	0.612
	Silvering stage II	0.035	0.097	-0.361	0.720
	Silvering stage III	-0.075	0.080	-0.944	0.351
	Parasitized x Silvering stage II	-0.050	0.134	-0.379	0.707
	Parasitized x Silvering stage III	0.134	0.127	1.057	0.297
Cortisol					
a) General					
	Time (1h)	0.315	0.050	6.260	<0.0001
	Time (4h)	0.095	0.050	1.881	0.062



Parasitized	-0.139	0.097	-1.428	0.159
Life-stage (silver)	0.117	0.073	1.601	0.115
Time (1h) x Parasitized	0.255	0.078	3.281	0.001
Time (4h) x Parasitized	0.139	0.078	1.783	0.077
Parasitized x Life-stage (silver)	-0.246	0.115	-2.140	0.037

b) Females in different silvering stages

Silvering stage II	-0.069	0.087	-0.791	0.433
Silvering stage III	0.142	0.082	1.717	0.093
Parasitized	-0.285	0.091	-3.120	0.003
Time (1h)	0.299	0.063	4.731	<0.0001
Time (4h)	0.095	0.063	1.509	0.135
Time (1h) x Parasitized	0.325	0.093	3.498	0.0008
Time (4h) x Parasitized	0.188	0.093	2.014	0.047

Na<sup>+</sup>/K<sup>+</sup>-ATPase activity

a) General

Sex (males)	2.717	0.755	3.599	0.0006
Parasitized	1.051	0.972	1.082	0.284
Life-stage (silver)	6.046	0.818	7.395	<0.0001
Parasitized x Life-stage (silver)	-3.506	1.308	-2.681	0.0095

b) Females in different silvering stages

Silvering stage II	1.973	0.937	2.106	0.0412
Silvering stage III	5.163	0.874	5.908	<0.0001
Parasitized	0.211	0.758	0.278	0.782

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Figure 1

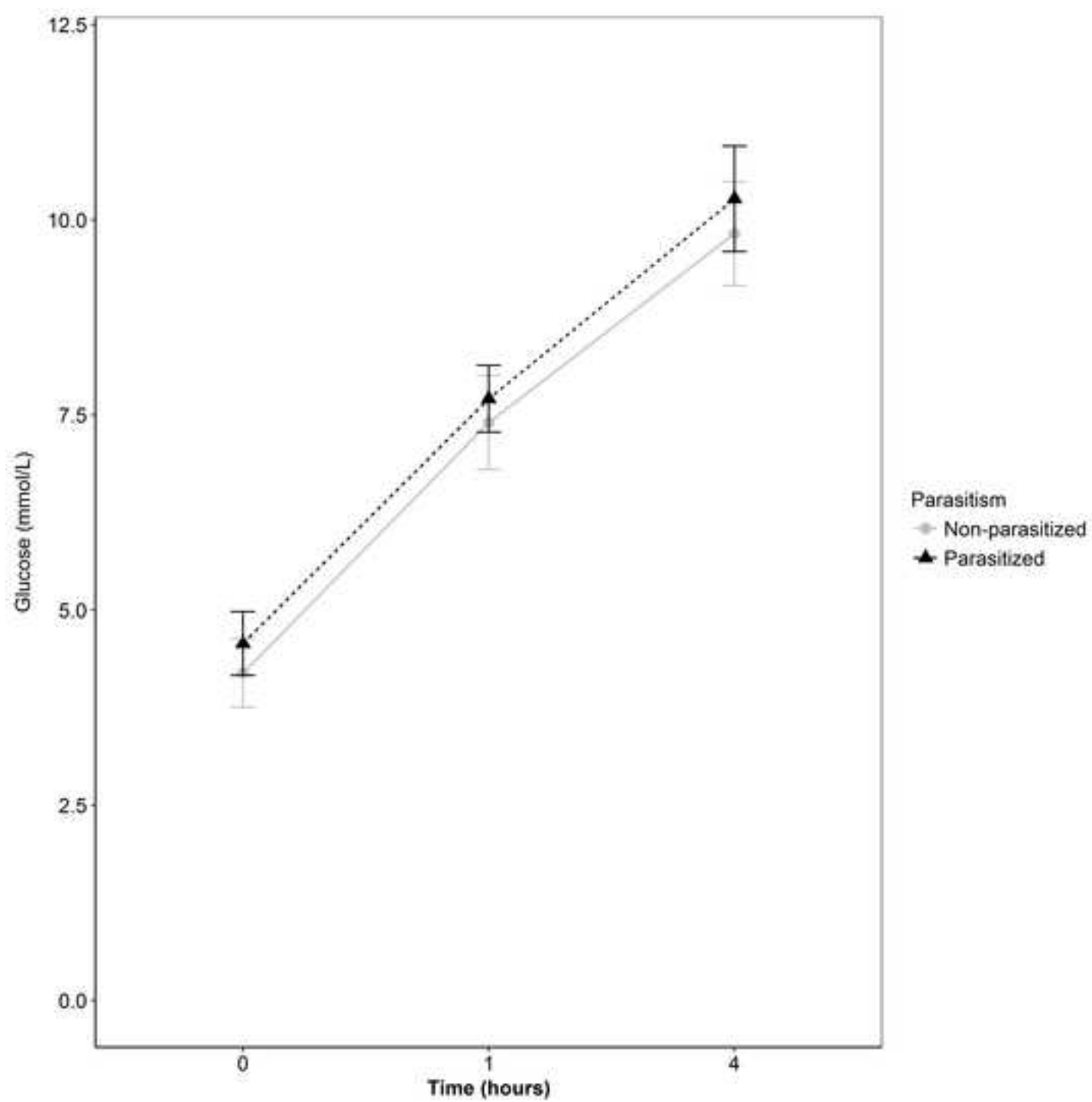


Figure 2a

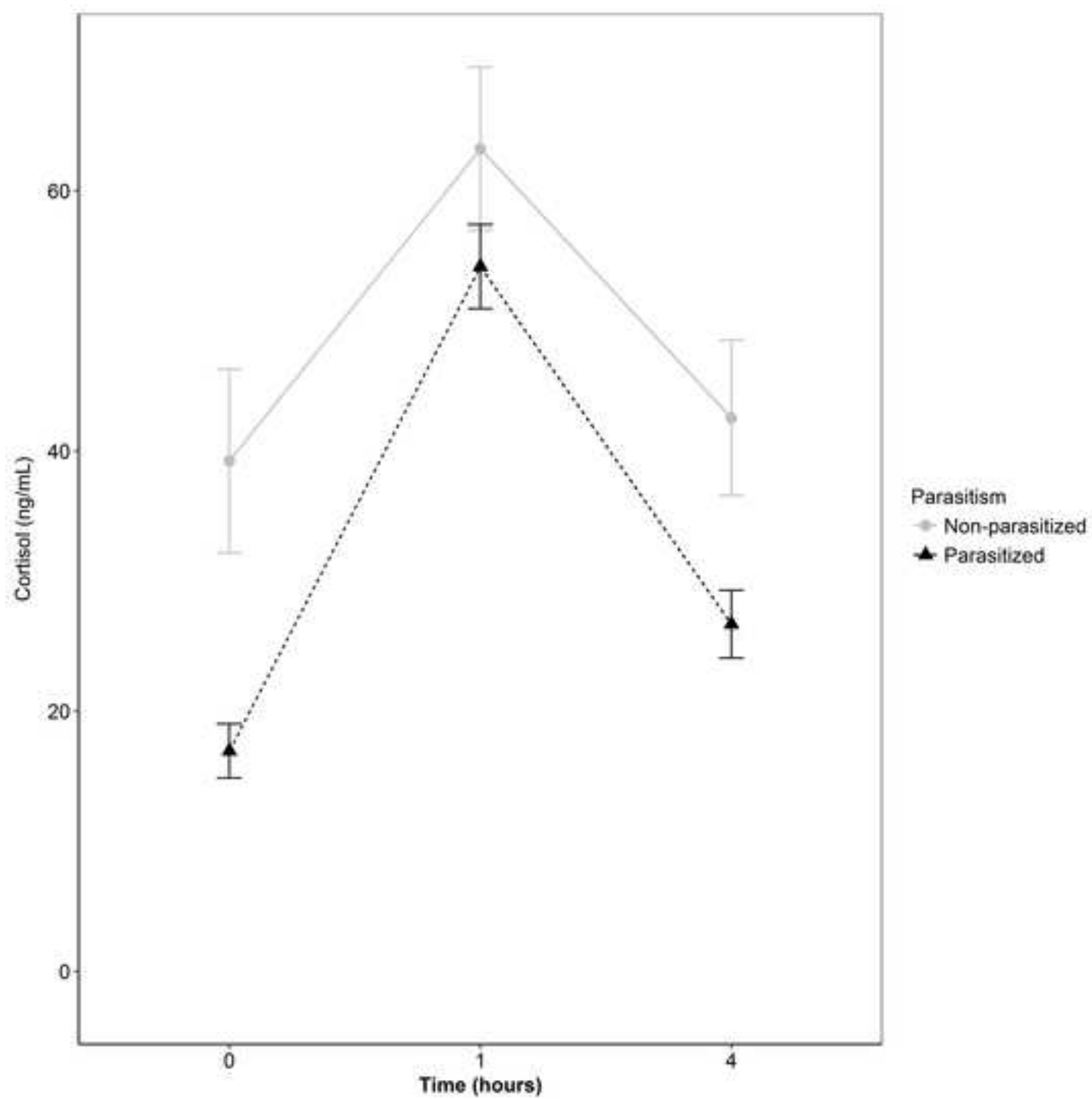


Figure 2b

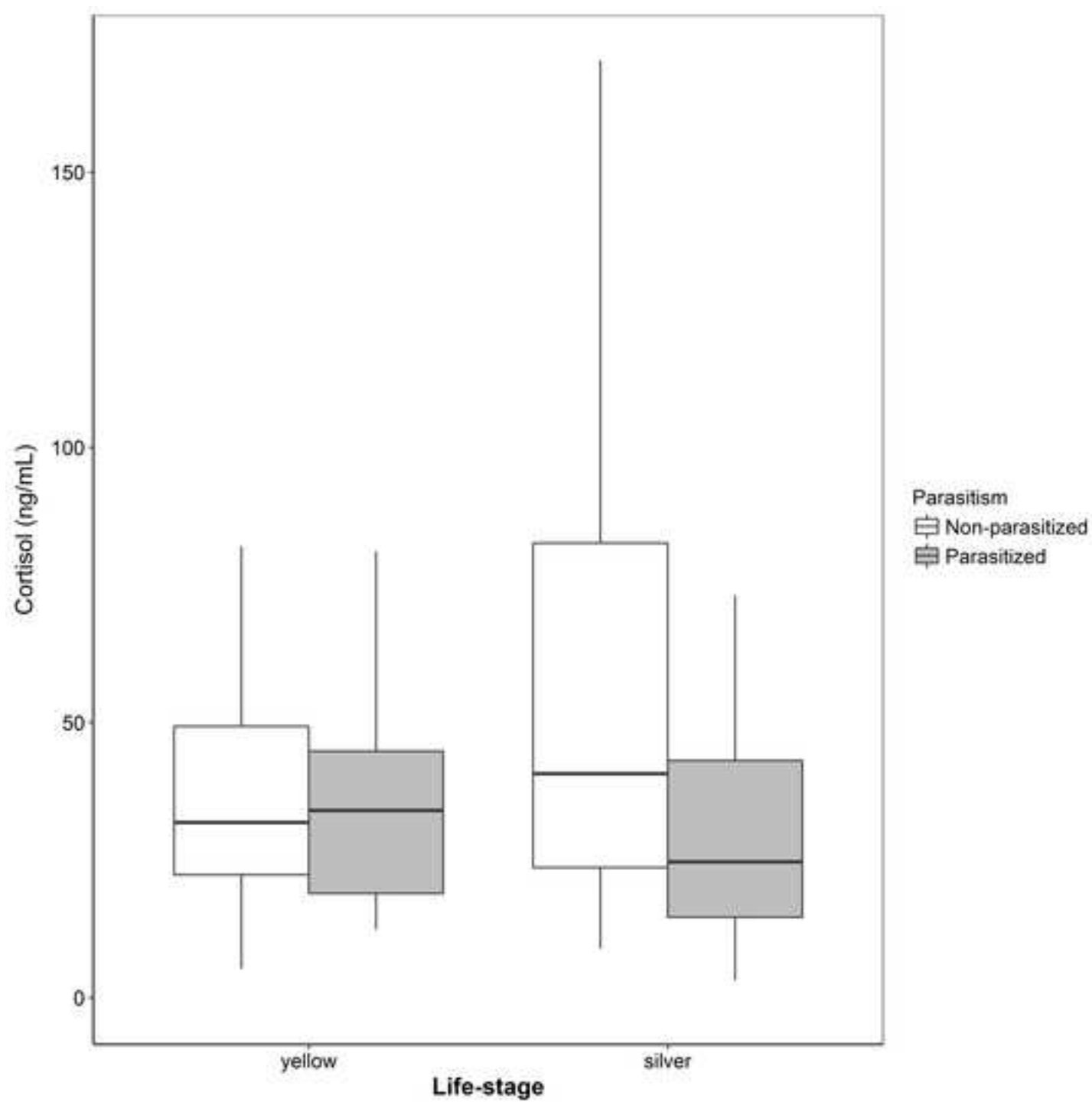


Figure 3

